Vitamin A&E in serum/plasma

1080 M VAE

Instructions for use, LC-MS/MS assay

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CE IVD Medical Device

Contents

1. General Information

1.1 Information for the Device

1080 M VAE - Vitamin A & E reagent set
UDI-DI: 872051431VAEDW
ENDM: W01019099 (see section 2.4 and 2.5 for ENDM codes individual products)

For information on the individual components of this set, refer to chapter 2 of these instructions for use.

1.1.1 IVDR classifications

Class B (not self-test/near patient test/companion diagnostic), based on rule 6; (EU) 2017/746, Annex VIII, 2.6.

IVR 0608
IVP 3002 / 3003
IVD 4002
IVS 1004 / 1006
IVT 2006

1.2 Manufacturer

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1.3 Intended Purpose

1.3.1 Measurand

Vitamin A
All-trans-Retinol (Vitamin A1)
(Retinol)

Vitamin E
Alpha-Tocopherol
(5,7,8-Trimethyl-tocol)

1.3.2 Function

This in vitro diagnostic medical device is intended for the quantitative determination of vitamin A (retinol) and/or vitamin E (alpha-tocopherol) in human serum or plasma. The results may assist healthcare professionals in assessing whether a patient has a deficiency, sufficient levels, or excess of one or both vitamins.
The method has been validated using serum-based calibrators and controls. While use with plasma may be feasible, it is the responsibility of the end user to verify the suitability of plasma samples under their specific conditions of use.

1.3.3 Required Specimen

Human serum is the validated specimen type.
Use with plasma may be possible, subject to user verification.

1.3.3.1 Conditions for collection, handling and preparation of specimen

This kit is designed using calibrators and controls based on human serum. As such, the method has been validated for use with serum samples.

While use with plasma may be feasible, the end user is responsible for confirming matrix equivalence and verifying method suitability for plasma under their specific conditions, particularly with respect to sample collection tubes, anticoagulants, and sample stability.

Internal comparison data demonstrated equivalent analytical performance of serum, EDTA plasma and heparin plasma under controlled conditions, with all matrices meeting defined accuracy thresholds. Nevertheless, due to possible variation in pre-analytical handling, users must verify matrix suitability within their own workflow.

The procedures for sample collection, handling, transport, storage, and preparation must be established and validated by the end user. Variability in pre-analytical factors — including blood collection materials and processing timelines — may influence analytical outcomes and are not within the scope of the manufacturer's validation.

It is therefore the responsibility of the user to ensure appropriate validation of all steps related to specimen integrity and compatibility with this kit, in accordance with their laboratory's quality management system and applicable regulations.

1.2.6 Testing Population

Patients suspected of having deviating levels of vitamin A or vitamin E.

1.3 Intended User

Laboratory Professional with basic understanding of LC-MS/MS.

1.4 Test Principle

1.4.1 LC-MS by ESI and MRM

Vitamin A (Retinol) and Vitamin E (Alpha-tocopherol) are determined from human serum or plasma using UHPLC coupled with positive ion electrospray LC-MS/MS.
Prior to LC-MS/MS analysis, a sample clean-up is performed to precipitate proteins and introduce isotope-labelled internal standards. This step minimizes matrix interference and ensures quantification accuracy.
After chromatographic separation on a C18 analytical column, Vitamin A and Vitamin E are ionized using electrospray ionization (ESI), a soft ionization technique that generates charged analyte molecules in the liquid phase before introducing them into the mass spectrometer. The generated ions enter a tandem mass spectrometer consisting of three quadrupoles: the first isolates the precursor ion, the second serves as a collision cell, and the third selects the resulting product ion for detection.
Measurement is performed in Multiple Reaction Monitoring (MRM) mode. In this mode, only ions with a defined mass-to-charge (m/z) ratio are selected in the first quadrupole. These ions are fragmented in the collision cell using an inert gas such as argon or nitrogen. Only product ions with the pre-defined m/z values pass the third quadrupole to be detected, ensuring highly selective identification and quantification of Vitamin A and Vitamin E.

1.4.2 Function of the Internal Standard Without a Specific Value

The internal standard solution must be added in the same volume to each calibrator, control, and patient sample. This ensures that every sample has the same internal standard concentration. The specific concentration of the internal standard is not relevant.
The internal standard compensates for interference (suppression) or enhancement from the matrix background. Since the patient matrix, calibrator matrix, and control matrix can all differ, the internal standard response will vary between samples. Because the internal standard has a very similar chemical nature to the analyte of interest, its signal will be suppressed or enhanced in a comparable manner.
When setting up the LC-MS software for quantitative analysis, it is important to assign a specific internal standard to each analyte and to set its concentration to 1.
The internal standard should ideally have a retention time as close as possible to that of its corresponding analyte.
It is also crucial to ensure that the internal standard is added to each sample in a consistent manner. Any variation in solubility, concentration changes over time (e.g., due to solvent evaporation), or differences in the time between sample preparation and measurement could lead to changes in concentration, potentially causing falsely elevated or decreased results. Strict adherence to the method is therefore essential.

1.5 Clinical Background

Vitamin A

Vitamin A deficiency is associated with a number of symptoms, of which xerophthalmia is nearly pathognomonic (1). In the Western world, where nutrition is plentiful, vitamin A deficiency is rarely seen. However, pregnant women (2) and patients suffering from malabsorptive disease (for example due to intestinal, pancreatic or liver pathology) vitamin A deficiency can occur in a large number of cases (3,4). Current guidelines for the prevention

and treatment of vitamin A deficiency have been combined into the World Health organisation Vitamin A supplementation guideline (5).
Detection of vitamin A deficiency is often based on an eye exam, supplemented with laboratory measurements if possible. In this, multiple biomarkers have been suggested and liver vitamin A reserves remain the gold standard (6). For obvious reasons, liver vitamin A content is not a preferred method of choice if a patient presents with symptoms that fit vitamin A deficiency. Instead, circulating vitamin A, vitamin A dose response* and isotope dilution assays have been developed.
Of these surrogate markers, a single measurement of circulating vitamin A levels require the lowest degrees of intervention but is (due to it's homeostatic control) known to be reduced from normal only if liver stores are greatly diminished (6). Also, in order to ensure an adequate interpretation, patients need to be clear from infection and/or inflammation due to the fact that retinol binding protein is an acute phase marker (7). In addition, due to the impact of diet, patients should refrain from high vitamin A diets prior to phlebotomy to prevent false high results and be fasted when there is doubt on their vitamin A status (8).
To date, there is no reference measurement procedure for vitamin A listed in the Joint Committee for Traceability in Laboratory Medicine database (accessed 4/7/2021) although laboratory best practice guidelines have been set up (8) and an LC/MS-MS assay has been proposed as a candidate reference measurement method (8). In this, it is good to realise that "vitamin A" encompasses the spectrum of compounds that exert vitamin A activity (9), even though measurements are commonly restricted to the predominant form: retinol, encompassing both the cis-, and trans-isomers.
In addition to aforementioned pre-analytical considerations, some analytical aspects are also crucial to ascertain correct measurement. For example, samples should be protected from oxidative destruction to ensure retinol is not inactivated. Also, as retinol is heat sensitive; no temperatures above 40 degrees should be used. A full list of (pre)-analytical considerations is beyond the scope of this work but can be found in this review (8).
In addition to aforementioned (pre)-analytical considerations, one must ascertain that the reference range provided by the manufacturer fits the population studied. Large differences in reference intervals have been reported, with ranges differing as much as 1.6 – 2.3 µM to 1 – 4 µM in laboratories employing HPLC.
*Notably, the aforementioned dose-response test also relies on serum vitamin A measurements but is sampled twice (before and after a high vitamin A dose) whereas the isotope dilution relies on the measurement of a stably labelled form of vitamin A.

Vitamin E

Vitamin E is a fat soluble vitamin, encompassing eight forms. These eight forms differ from each other by differential methylation of a chromane head group (10). From these, only alpha-tocopherol is regulated in human plasma and this form is measured to investigate dietary sufficiency, which is debatably set to a plasma/serum concentrations above 12 µM (10,11).
Vitamin E deficiency is associated with a number of symptoms. Primary vitamin E deficiency (familial vitamin E deficiency due to mutations in the a-TTP gene; AVED) is associated with neurological symptoms and ataxia (12) and is diagnosed on molecular level. Secondary vitamin E deficiency is rare in the Western world, and mostly seen in patients suffering from altered fat metabolism. In patients suffering from fat malabsorption, vitamin E levels can drop to deficient levels and symptoms mimicking primary deficiency can develop albeit often milder (12). Secondary vitamin E deficiency is based on symptomology and diagnosed based on plasma/serum alpha-tocopherol measurement (11).
Alpha-tocopherol does not rise significantly in blood after a standard meal as it's abundance is low in plants (in contrast to the high concentration of gamma-tocopherol found (13,14)). However, co-ingested with a high fat meal it does increase, and the immediate postprandial

bioavailability is higher in such situations (14). Therefore, measurements of alpha-tocopherol should not take place after consumption of a high fat, alpha-tocopherol enriched meal. Also, one should consider correcting alpha-tocopherol levels for blood lipid levels although this seems necessary only in patients suffering from grossly altered lipid metabolism (15,16).
To date, there is no reference measurement procedure for vitamin A listed in the Joint Committee for Traceability in Laboratory Medicine database (accessed 10/7/2021) although laboratory best practice guidelines have been set up (13) and an LC/MS-MS assay has been proposed as a candidate reference measurement method (17).
In addition to aforementioned pre-analytical considerations, some analytical aspects are also crucial to ascertain correct measurement. In general, vitamin E is more stable in chilled blood but is not light sensitive. A full list of (pre)-analytical considerations is beyond the scope of this work but can be found in this review (13).
In addition to aforementioned (pre)-analytical considerations, one must ascertain that the reference range provided by the manufacturer fits the population studied. Large differences in reference intervals have been reported, with ranges differing as much as 7.5 – 30 µM to 19 – >50 µM in laboratories employing HPLC.

1.6 Notice Regarding Serious Incidents

Following (EU) 2017/746 Annex I, Chapter III, 20.4.1 af), any serious incident that has occurred in relation to this device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

1.7 IVD symbols

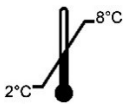
Order Number

Lot Number

For in vitro diagnostic use


See instructions for use


Manufacturer



Temperature limits

Contains sufficient for < n > tests


Expiry date

2. Components and Accessories

2.1 Safety information

1 Before starting the assay, ensure that you have thoroughly read and fully understood the instructions.

2 Some components are chemical preparations and may contain hazardous substances. For safety information, please refer to the Material Safety Data Sheet (MSDS) for each component.

3 The donor blood used as raw material was tested for HBsAg, anti-HIV 1/2, and anti-HCV. However, since no test method can guarantee that products derived from human sources are completely free from infectious agents, it is recommended to handle this product with the same precautions as patient samples.

4 Dispose of all reagents as hazardous waste in accordance with your national biohazard and safety guidelines or regulations. It is important to ensure that everyone who may come into contact with the products is informed about the proper handling and disposal procedures.

5 Any serious incidents related to the device must be reported to the manufacturer and the relevant competent authority.

2.2 Storage conditions and lifetime of kit components

1 Upon receipt, immediately unpack the kit components from the transport packaging and follow the storage instructions indicated on the product labels.

2 If any component is damaged, collect physical evidence (e.g., by taking a photo) and contact Diagnotix within one week. Do not use any damaged product and store it in a safe location.

3 Handle broken glass with care to prevent injury.

4 Do not use expired products. Do not mix reagents of the same type.

2.3 Description of Components

All components are intended for LC-MS/MS use only. They may also contain ingredients other than the active ingredients listed below, which could influence the measurement. All stated stability conditions are only valid in the absence of bacterial contamination.

2.3.1 Calibrators and Controls

2001 CAL M VAE | Vitamin A & E Calibrator Set
UDI: 8720514310922

A six-point lyophilized human serum calibrator at clinically relevant levels, refer to the value data sheet provided with each set for specific values per production batch.

2002 CON M VAE | Vitamin A & E Control Set
UDI: 8720513410939
2012 M VAE | Vitamin A & E Control I
UDI: 8720514311035
2012.10 M VAE | Vitamin A & E Control I (10-pack)
UDI: 8720514313541
2013 M VAE | Vitamin A & E Control II
UDI: 8720514311042
2013.10 M VAE | Vitamin A & E Control II (10-pack)
UDI: 8720514313558
2014 M VAE | Vitamin A & E Control III
UDI: 8720514311059
2014.10 M VAE | Vitamin A & E Control III (10-pack)
UDI: 8720514313565

Three levels of lyophilized human serum controls at clinically relevant levels for quality control purposes, refer to the value data sheet provided with each set for specific values per production batch.

2.3.1.1 Handling

Reconstitute the calibrators and controls as follows:

1. Carefully remove the cap and rubber plug avoiding any loss of contents.
2. Reconstitute Vitamin A & E Calibrator Set and Controls with exactly 500 µl distilled or deionised water using a volumetric pipette.
3. Reseal the vial with the plug and let stand during 15 minutes.
4. Swirl the vial carefully and mix thoroughly making sure that all traces of dry material have dissolved, do not shake. Avoid foaming.
5. Let stand for 15 minutes at room temperature.
6. Swirl the vial carefully, do not shake. Avoid foaming.
7. Use the preparation as a patient sample.

2.3.1.2 Stability and storage

The stability of the calibrators and controls are:

Before reconstitution:	2 - 8 °C	Until expiry date printed on the product label
After reconstitution:	2 - 8 °C	8 days
After reconstitution:	- 20 °C	1 month

Maximum of one freeze-thaw cycle is allowed.
The declared stated stabilities are only valid in case of no (bacterial) contamination.

2.3.1.3 Metrological Traceability

Metrological traceability is established by comparing each batch to the highest available order of reference material, as well as the last batch produced before the current batch.

For Vitamin A & E the highest available order of reference material has been established to be the reference laboratory network of the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML).

Refer to the Metrological traceability sheet for the specific batch for more information.

2.3.2 Deproteinization Solution with Internal Standard

1090 M VAE | Vitamin A & E Deproteinization Solution with Internal Standard
UDI: 8720514311004
1090.10 M VAE | Vitamin A & E Deproteinization Solution with Internal Standard (10-pack)
UDI: 8720514313534

A deuterated version of the measurand, dissolved in a deproteinization solution. Used to identify and correct potential deviating values, due to errors or varying circumstances in sample preparation or within the LC-MS.
The Deproteinization Solution removes proteins from the sample. These proteins form a pellet after centrifugation.

2.3.2.1 Handling

The Reagent is liquid and ready for use.

2.3.2.2 Stability and Storage

The stability of the Deproteinization with Internal Standard is:

Before opening: 2 - 8 °C	Until expiry date printed on the product label
After opening: 2 - 8 °C	2 weeks
After opening: -20 °C	1 month

The declared stated stabilities are only valid in case of no (bacterial) contamination.

Active ingredient(s):
Thiamine pyrophosphate-D3 & Pyridoxal-5'-phosphate-D3.

Acetonitrile	75% - < 100%
Methanol	10% - < 25%

2.3.3 Mobile Phases

1091 M VAE | Vitamin A & E Mobile Phase I
UDI: 8720514311011
1092 M VAE | Vitamin A & E Mobile Phase II
UDI: 8720514311028

Two mobile phases are added to tune and carry the sample through the LC-MS/MS. Different ratios of the mobile phases will allow different components to eluate from the column at differing speeds.

Active ingredient(s):

Mobile Phase I:	75% - <100% Water
Mobile Phase II:	75% - <100% methanol

2.3.3.1 Handling

The Reagents are liquid and ready for use.

2.3.3.2 Stability and storage

Store at 2 - 8 °C	After first opening the Reagent can be used for 6 weeks if closed and stored at 2 - 8 °C or 2 weeks on the UHPLC
Store at RT	Before first opening the Reagent can be stored for 6 weeks at Room Temperature

The declared stated stabilities are only valid in case of no (bacterial) contamination.

2.3.4 Autosampler Washing Solution

1096 M VAE | Vitamin A & E Autosampler Washing Solution
UDI: 8720514310199

A solution used to clean the LC-MS/MS system after use, specifically designed to remove residue from testing the measurand.

Active ingredient(s): Acetonitrile 50% - <75%

2.3.4.1 Handling

The Reagent is liquid and ready for use.

2.3.4.2 Stability and storage

Store at 2 - 8 °C	After first opening the Reagent can be used for 6 weeks if closed and stored at 2 - 8 °C or 2 weeks on the UHPLC
Store at RT	Before first opening the Reagent can be stored for 12 weeks at Room Temperature

2.4 List of components provided

1080 KIT M VAE - Vitamin A & E Reagent Set
EMDN code: W01019099

Contents (for 300 assays):

Description	Part number	Content	EMDN Code
Vitamin A & E Calibrator Set (Calibrator 1 – 6)	2001 CAL HM VAE	6 x 2 x 500 µl	W0101050301
Vitamin A & E Deproteinization Solution with Internal Standard	1090 M VAE	3 x 100 ml	W0101050399
Vitamin A & E Mobile Phase I	1091 M VAE	1 x 250 ml	W01019099
Vitamin A & E Mobile Phase II	1092 M VAE	1 x 500 ml	W01019099
Vitamin A & E	1096 M VAE	1 x 1000 ml	W01019099

Autosampler Washing Solution			
Vitamin A & E Manual			

2.5 Separately available materials and components

Vitamin A & E Calibrator Set (Calibrator 1 – 6)	2001 CAL HM VAE	6 x 2 x 500 µl	W0101050301
Vitamin A & E Deproteinization Solution With Internal Standard	1090.10 M VAE	10 x 100 ml	W0101050399
Vitamin A & E Mobile Phase I	1091 M VAE	1 x 250 ml	W01019099
Vitamin A & E Mobile Phase I	1091.5 M VAE	1 x 125 ml	W01019099
Vitamin A & E Mobile Phase II	1092 M VAE	1 x 500 ml	W01019099
Vitamin A & E Mobile Phase II	1092.5 M VAE	1 x 250 ml	W01019099
Vitamin A & E Autosampler Washing Solution	1096 M VAE	1 x 1000 ml	W01019099
Vitamin A & E Autosampler Washing Solution	1096.5 M VAE	1 x 500 ml	W01019099

Vitamin A & E Control I	2012.10 HM VAE	10 x 500 µl	W0101050101
Vitamin A & E Control II	2013.10 HM VAE	10 x 500 µl	W0101050101
Vitamin A & E Control III	2014.10 HM VAE	10 x 500 µl	W0101050101
Vitamin A & E Control Set (Control I – III)	2002 CON M VAE	3 x 3 x 500 µl	W0101050101
10 µg/ml solution Retinol (RUO)	TS-1081	1 ml	W0101050399
10 µg/ml solution Retinol-D6 (RUO)	TS-1081-I	1 ml	W0101050399
10 µg/ml solution Alpha-Tocopherol (RUO)	TS-1082	1 ml	W0101050399
10 µg/ml solution Alpha-Tocopherol-D6 (RUO)	TS-1082-I	1 ml	W0101050399

2.6 List of required additional products not supplied

Category		**Examples**
General laboratory equipment	UHPLC-MS/MS	Sciex 4500 or higher Shimadzu LC8050 or higher Waters TQS-µ or higher
	Vortex shaker	VWR VV 3
	Calibrated pipette 20-200 µl	
	Calibrated pipette 100-1000 µl	
	2-8°C controlled refrigerator	

	-20°C controlled freezer	
	Centrifuge	Eppendorf Centrifuge 5425
	Tube roller	Phoenix instrument RS-TR05
General laboratory material	Sample tubes	
	Autosampler vials, inserts, caps or well plates	
	Pipette tips 20-200 µl	
	Pipette tips 100-1000 µl	
	Analytical column	See section 4.1.6

3. Warnings, precautions, measures and limitations of use

3.1 General

The device and its components must only be used in line with the intended purpose by the intended user as stated in chapter 1. Due to their nature, most reagents of this device contain or are largely composed of hazardous substances. Please refer to the Safety Data Sheets (SDS) for each of the components for specific hazards and measures to be taken.

Used components should be discarded and are not suitable for re-use.

3.1.1 Potentially infectious material

The human material used for manufacturing calibrators and controls was tested for various markers for infectious diseases and/or pathogens and found negative. Nevertheless, the Whole blood calibrators & controls should be considered as potentially infectious and treated with appropriate care.

3.2 Interferences & Limitations

Visual evidence of lipemia, homolysis, or icterus (hyperbilirubinemia) and/or older age of the specimen may affect the performance of the device.

3.3 CMR substances

Methanol is present in the deproteinization solution. Methanol has been classified as a Category 2 reproductive toxicant according to Annex VI of Regulation (EC) No. 1272/2008. Although not classified as carcinogenic or mutagenic, methanol may pose a risk to human reproduction and is suspected of causing harm to the unborn child.

3.4 Disposal

For the safe disposal of the components of this kit, please refer to the safety data sheet of the component in question.

4. Assay procedure

4.1 System suitability & Method installation

4.1.1 Minimum requirements instrument and LC modules

Using this test kit requires a UHPLC system with tandem mass spectrometer (LC-MS/MS) with the following modules:

- Autosampler
- UHPLC gradient pump
- Column heater
- Degasser
- Proper gas supply is required for the specific MS/MS system in use

4.1.2 Minimum requirements tandem mass spectrometer

The tandem mass spectrometer should be sensitive enough to achieve a signal-to-noise ratio of at least 10 for calibrator 2.

To the best of our knowledge, no two LC-MS/MS systems react exactly the same due to minor technical differences between manufacturers, sensitivity, age, time of usage, location settings, and individual machinery conditions. Therefore, the MS settings, including the m/z transitions mentioned in section 5.4.3, should be considered as a guideline. It is strongly recommended that users tune and optimize their instrumentation as part of the method installation.

4.1.3 Minimum requirements UHPLC

The UHPLC should have a backpressure limit of at least 600 bar. It is strongly recommended to use a binary pump.

4.1.4 Minimum requirements chromatography

Due to minor differences between UHPLC and MS devices, the chromatographic conditions, such as temperature, gradient, and injection volume, may need to be adjusted to ensure the following conditions are met:

- A conditioned column prior to use (see 4.1.5)
- A minimum retention time of 3 column volumes
- Recommended retention time for vitamin A around 2 minutes
- A resolution of at least 1 between Vitamin A and E, but advised is at least 1.5 (baseline separation)
- Repeatable retention times for both Vitamin A and Vitamin E between runs. If retention times fluctuate by more than 0.2 minutes, this may indicate that the gradient is not properly set or the column is not adequately conditioned.
- For quaternary pumps, it is advisable to extend the reconditioning step of the gradient to improve the repeatability of retention times.

4.1.5 Column conditioning and maintenance

In most cased the analytical column is ready to use.

he column should be stored in the storage solution recommended by the manufacturer. Before reuse after storage, it may be necessary to flush the column with the initial gradient conditions until a stable backpressure is achieved, ensuring complete removal of the storage solvent. Store the column in its original box, in a dark, temperature-controlled environment, protected from continuous movement and vibrations.

4.1.6 Examples of suitable columns

The list below contains columns that Diagnotix has used or is using for this method. However, other columns may also suffice, as long as the minimum chromatography requirements as stated in section 4.1.4 are met.

Supplier	**Product name**	**Part number**
Waters	Xbridge BEH C18 XP Column 2.5 µm, 3mm x 75 mm	186006034
Waters	XSelect BEH C18 2.5 µm 2.1mm x 75 mm	186006102
Phenomenex	Luna Omega Polar C18 1.6 µm 50 x 2.1 mm	00B-4748-AN

4.1.7 Verification & Validation

We recommend that users perform at least a verification of the method using independent controls and correlation studies. A full validation is advised due to potential LC-MS/MS system-specific variations.

A comparison study with an existing method is highly recommended. Diagnotix offers the possibility to conduct comparison studies. Please contact us for more information.

Additionally, we recommend incorporating multiple-level controls in each batch of runs and participating in proficiency testing programs. Diagnotix provides three levels of lyophilised whole blood controls (low, medium, and high), which are available separately from the reagent kit. These controls are not an integral part of the reagent set, to allow laboratories the flexibility to use fully independent, third-party control materials where required. This supports unbiased internal validation and is aligned with best practices for quality assurance and regulatory expectations.

Using multiple control levels is important to verify calibration accuracy across the entire clinically relevant measuring range. Controls should be selected to represent low-end, mid-range, and high-end concentrations, ensuring the analytical system performs reliably at all points on the calibration curve.

In addition, it is recommended to repeat control measurements at regular intervals during large sample series. This allows the early detection of drift, matrix interferences, or technical variation over time. Control tracking supports stability monitoring and ensures timely corrective actions can be taken before patient results are affected.

The routine use of matrix-matched control materials is a cornerstone of laboratory quality assurance. It improves result traceability, supports conformity with regulatory requirements, and enables performance verification over time.

Diagnotix strongly recommends implementing such control strategies within a quality management system in line with ISO 15189. This includes maintaining documented procedures for control evaluation, trend analysis, and root cause investigation. Participation in proficiency testing schemes (external quality assessment, EQA) is also advised to ensure inter-laboratory comparability and long-term analytical robustness.

4.1.8 Installation requirements

Diagnotix or its partners can be consulted to assist with or perform the installation of this kit on your LC-MS/MS device. Please contact Diagnotix for more information.

We strongly recommend that the method installation be performed by a skilled staff member who is familiar with your LC-MS/MS device, software, data analysis, and the general principles of LC-MS/MS analysis for clinical applications. The staff member should, at a minimum, be able to tune the system, evaluate and optimize the gradient, and generate and interpret data.

4.1.9 General Advice for Gradient Management

This section provides a general explanation. There may be method-specific requirements which can be found in the section *System Suitability & Method Installation*. In case of doubt, please contact Diagnotix at techsupport@diagnotix.com or consult your officially supplying partner.

Although Diagnotix kits specify fixed compositions for Mobile Phase I and II, the gradient profile may be adapted to suit the specifications of the LC system in use, provided that critical functional requirements are maintained.

Due to the complexity of human matrices, it is crucial to adhere strictly to general gradient principles. LC-MS/MS is an excellent tool for detecting and isolating molecules of interest; however, one should not underestimate the presence of other molecules within the sample. These can affect reproducibility, sensitivity, and overall system suitability.

It is important to note that gradients are typically designed by increasing or decreasing the percentage of Mobile Phase II, which is the organic component. Correspondingly, Mobile Phase I is the aqueous (water-based) component.

Generally speaking, all proper gradients consist of the following parts: a beginning phase starting at a low percentage of organic solvent; an elution phase as a linear increase towards high organic; a washing phase to wash off strongly retained compounds; and a conditioning phase, which returns to the initial low percentage organic either linearly or immediately.

Beginning phase
This phase is intended to wash off highly polar molecules (such as sugars, salts, etc.). When analysing relatively hydrophobic compounds, one can choose to increase the starting organic percentage, enabling the removal of more unwanted molecules.

Elution phase
In this phase, the percentage of organic solvent is increased to allow the analytes of interest to elute. The elution phase must be designed to ensure the method remains reproducible and validated. Complete chromatographic separation of compounds is not a necessity for LC-MS/MS, although it can help improve sensitivity. Isobaric compounds, however, must be

baseline separated, as MS/MS detection cannot distinguish between them due to their identical mass-to-charge ratios.

Washing phase
The washing phase is extremely important for removing highly hydrophobic molecules that may adhere to the column or system and require sufficient time to dissolve in the mobile phase. These molecules can influence reproducibility and sensitivity over time and may interfere with other methods if they remain in the system. It is advisable to wash for at least five column volumes—more if possible. The flow rate may be increased to reduce washing time, if the system and column can tolerate the increased backpressure. Besides the washing step after each injection, it is strongly recommended to routinely wash the system with high organic content during runs to prevent contaminant build-up.

Conditioning phase
It is important that all injections start under identical conditions. Therefore, it is crucial to ensure that the entire system returns to the starting conditions by washing with the initial gradient at the end of each run. Ideally, this step should last for at least 6–10 column volumes, depending on the LC pump's performance and the system's dwell volume. These values will differ between LC-MS systems, laboratories, and with system ageing.

Calculating column volume
To determine your column's volume, consult the column manufacturer. As a rough estimate, consider the column as a cylinder and calculate its total volume. For columns packed with fully porous material, 60% of the total volume is a reasonable estimate of the actual column volume. For core-shell material, this would be 50% of the total cylinder volume.

4.1.10 Other method specific parameters to consider

This section provides a general explanation. There may be method-specific requirements which can be found in the section *System Suitability & Method Installation*. In case of doubt, please contact Diagnotix at techsupport@diagnotix.com or consult your officially supplying partner.

Injection mode
In pre-injection gradient start, the gradient begins before injection; dwell volume must be accounted for to ensure correct elution timing. In post-injection gradient start, the gradient begins after injection. This can improve reproducibility for early eluting compounds but requires careful timing to avoid band broadening or peak distortion.

Injection volume
Decreasing the injection volume can reduce matrix contamination and improve peak shape for abundant compounds. Increasing the injection volume may enhance sensitivity and reduce the risk of background noise being misinterpreted as part of the analyte peak. When analysing compound panels, it is advised to reduce the injection volume to ensure that the most sensitive peak remains clearly measurable. However, if this leads to overloading of high-abundance peaks, one may choose to down-tune the MS settings to reduce signal intensity.

Sample loop
The sample loop size must match the injection volume to prevent dispersion or loss. Typically, the injected volume should be 50–80% of the loop capacity. For small-volume injections, direct injection or bypassing the sample loop may prevent dilution in the mobile phase. Although dilution can negatively affect peak shape, this effect is usually negligible at moderate to high flow rates. Additionally, dilution may lower the organic content of the sample in the loop, potentially improving the peak shape of early eluting compounds.

Column temperature
It is recommended to maintain the specified column temperature as standard. Higher temperatures generally reduce backpressure and allow faster flow rates, though they may shorten column lifespan. It is essential not to exceed the manufacturer's temperature limits. Columns should always be temperature-controlled, as fluctuations between runs can affect chromatographic behaviour and reduce reproducibility. Some systems allow for pre-heating of the mobile phase prior to entering the column, which may help maintain consistent column temperature but is not strictly necessary with Diagnotix products.

Flow rate
Flow rate should align with column width and efficiency. Adjusting flow rate affects chromatographic performance but is not inherently problematic. Increasing the flow rate may shorten run time but raises backpressure, while decreasing it lowers backpressure. However, without adjusting gradient timing proportionally, changes in flow rate can affect chromatographic behaviour, so gradient adjustments are recommended. The relation between column material and optimal flow rate impacts peak shape and resolution. Though knowing the optimal flow rate is useful, operating under suboptimal conditions may be beneficial—for example, to reduce backpressure or shorten run times. Consistency between runs and within patient sample batches is most important, as is preventing carry-over by using appropriate washing and reconditioning steps.

Column length
Column length is proportional to efficiency. Longer columns allow for better separation and improved peak shape but also increase run time and backpressure. This limits the possibility of using higher flow rates or may reduce column lifespan. Conversely, shortening the column may improve throughput and reduce analysis time, provided that the loss of chromatographic resolution is acceptable.

Guard columns or column protection products
Diagnotix does not advise against the use of column protection products, such as guard columns. However, these can influence the dwell volume and chromatographic behaviour of the method. It is important to replace such products on time to avoid excessive backpressure or chromatographic disturbances during analytical runs.

Protein precipitation or phospholipid removal plates
Although Diagnotix has not validated the use of protein precipitation or phospholipid removal plates within the workflow of its methods (unless specifically stated), such tools may offer practical benefits during sample preparation. These plates allow the deproteinisation step to be performed without requiring a centrifugation step to collect the eluate. The user remains solely responsible for validation. As part of this validation process, a comparison study must be carried out using real patient samples across the entire clinically relevant concentration range.

4.2 The analytical system

4.2.1 Preparing the LC-MS system

- Use the solvents supplied with the kit.
- Flush the LC system to remove any residues or contaminants from previous analyses. You may use the mobile phases or autosampler Washing Solution provided in the kit for this purpose.
- Install and connect the analytical column, ensuring correct orientation and secure fittings.
- Set system parameters (flow rate, temperature, gradient) and equilibrate according to the intended method.
- Verify stable baseline and system pressure.

Note: Always follow the safety guidelines and operation manual for the LC-MS system. Method-specific conditions should be applied as validated in your laboratory.

4.2.2 Starting the analytical system

- Equilibrate the system.
- Check the temperature of the column.
- Initialize the injector.
- Start the programme on the LC-MS/MS system.
- Perform several test runs with an easy-to-measure standard (e.g. a standard with a sufficiently high concentration) until consistent and reproducible results are obtained (System Suitability Test).

4.3 LC-MS/MS Parameters and Conditions

4.3.1 LC Parameters

UHPLC pump	Flow rate 0.55 ml/min
Mobile Phases I and II	Close the bottles to avoid alteration of RT's through evaporation of the mobile phases
Column	The column is installed in the column heater 30°C For the complete UHPLC system the backpressure should not exceed 800 bar. 1 bar = 14.5 PSI

4.3.2 Autosampler Conditions

Injection volume:	2 µL
Sample temperature:	10 °C
Runtime:	4 min
Column temperature:	45 °C ± 2 °C alarm
Needle wash:	wash twice for 6 seconds
Seal Wash:	10:90 ACN:H2O
Wash Solvent:	Autosampler Washing Solution; 1096 M VAE

4.3.3 Gradient

Time (min)	**Flow Rate (mL/min)**	**%A**	**%B**	**Curve**
0.00	0.55	35	65	Initial
2.00	0.55	5	95	6
3.00	0.55	5	95	6
3.10	0.55	35	65	6
4.00	0.55	35	65	6

Please note that the gradient is dependent on the analyser used. End users will need to define the optimal gradient for the analyser in use.

4.3.4 MS Conditions (e.g. Waters Xevo TQS)

MS System:	(Waters Xevo TQS)
Ion mode:	Electrospray
Capillary voltage:	1.0 kV
Polarity:	positive
Source temperature:	150 °C
Desolvation temperature:	650 °C
Desolvation gas flow:	1000 L/hr
Detection mode:	MRM
Dwell time:	0.019 sec
Collision gas:	Argon / Nitrogen

Substance	**Precursor**	**Product**
Vitamin A	269.30	81.00
Vitamin A	269.30	93.00
Vitamin A D6	275.30	81.00
Vitamin A D6	275.30	93.00

Substance	**Precursor**	**Product**
Vitamin E	431.40	83.00
Vitamin E	431.40	165.10
Vitamin E D6	437.40	83.00
Vitamin E D6	437.40	171.10

These conditions are an indication, the optima can differ slightly between different LC-MS/MS systems.

5. Sample

5.1 Sample material

Human serum / plasma (EDTA- and Heparin-tubes)

Because sample collection, transport and storage conditions may differ per end-user**. End users are responsible for their own sample extraction and storage validation.**

Users are assumed to have read sections 1.3.3 and 1.3.3.1 and to be aware of any laboratory-specific risks linked to the chosen sample material and collection method.

5.2 Sample preparation

Please note: as reagent temperature can negatively impact the effectiveness of the Deproteinization Solution, Diagnotix strongly recommends using this reagent at the indicated temperatures and to not let the reagent warm up to room temperature before use.

5.2.1 Reconstitution of the lyophilised Calibrators / Controls.

See 2.3.1.1 and the product data sheets.

5.2.2 Sample preparation (patient sample, calibrator or control)

1. 25 µl sample, calibrator or control.
2. Add 975 µl Deproteinization solution with Internal Standard deuterated.
3. Mix immediately using a vortex mixer for 30 seconds.
4. Centrifuge (5 min, 10000 x g or more).
5. Transfer 400 µl centrifuged supernatant to a vial or 96 well plate, which is suitable for the auto sampler in use and Inject 2 µl in the LC-MS/MS.

5.2.3 Sample Preparation with pipette robot

Into a 2 ml 96 well plate:

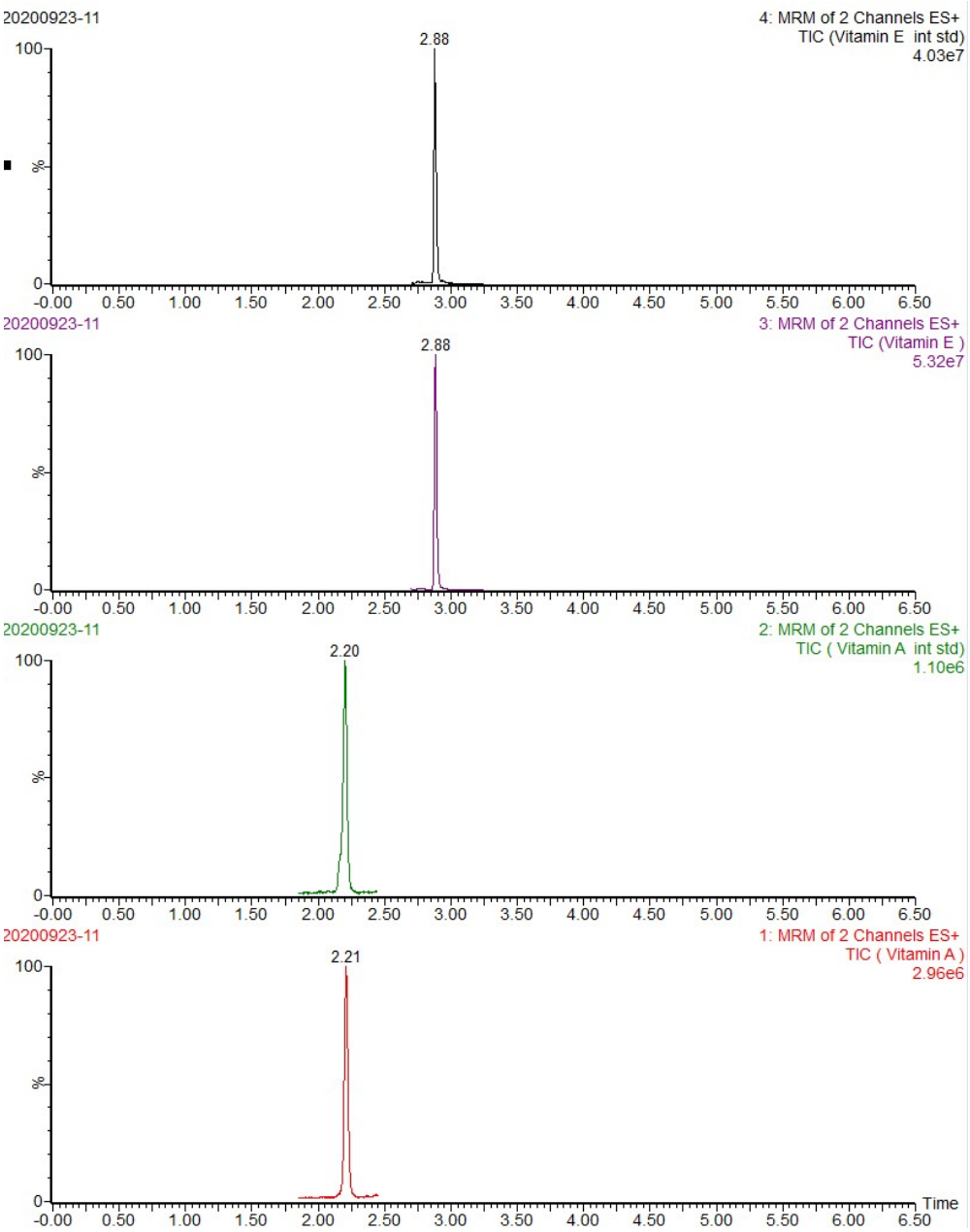
1. 25 µl sample (Calibrator, Control, Patient sample),
2. Whilst mixing the plate, add 975 µl Vitamin A & E deproteinization solution with Internal Standard.
3. Once mixing is complete, centrifuge (5 min , 10,000x g or more).
4. Transfer the samples into a 1 ml 96 well collection plate for injection on the UHPLC/MS/MS system (Needle placement 2 mm) or inject directly off the pellet (Needle placement 10 mm) 2 µl in the LC-MS/MS.

5.2.4 Autosampler stability

Autosampler stability at 15 °C for 72 hours indicates that vitamin A remains stable, with no notable change in concentration. Vitamin E, however, may show a decrease of up to 5%.

5.3 Examples of chromatograms

Example chromatogram of a Patient sample, recorded with the Waters LC-MS/MS TQS-µ:



5.4 Results from LC-MS/MS and Reference Values

5.4.1 Data acquisition

It is advised that users are fully trained onto using the supplied LC-MS/MS software and the concepts of performing quantify MS calculations.

Assign the concentration of the internal standard on 1.
Use a weighting factor of 1/x and do not force the calibration curve through zero.

with some systems, and in some cases, the lowest calibrator level may not be measured accurately. Therefore, evaluate your calibration curve both with and without calibrator 1, and choose the calculation that shows the least deviation and the best fit to your controls.

It is recommended to always review the chromatographic results of standards to ensure proper integration. Manually correct the integration if necessary.

Fragmentation Ratio Stability

Always monitor the fragmentation ratio for each compound. This ratio should remain stable under normal conditions. Any observed deviations may indicate contamination or a malfunctioning instrument.
If changes are detected:

1. **Do not use** the obtained results for data acquisition.
2. **Contact your service provider** immediately for further diagnosis and maintenance.

5.4.2 Reference values

The assay will result in a certain value for the measurand, which will need to be compared to applicable reference values to be interpreted for the specific patient.

Analyte	**Consensus reference interval (serum/plasma, adults)**
Vitamin A	1.0 – 4.2 µmol/l
Vitamin E	15 – 50 µmol/l

Note:
The inclusion of this information is required by Annex I, Section 20.4.1 (v) of Regulation (EU) 2017/746 (IVDR).
Reference intervals are provided for indicative purposes only. These consensus values were established by combining reference data from multiple ISO 15189-accredited laboratories in the Netherlands and verifying the absence of clinically significant bias in method-comparison studies.
Variations may occur due to methodology, population, and dietary factors. Each laboratory should establish and verify its own reference intervals in accordance with ISO 15189 requirements.
In addition, local laboratory conditions—such as sample handling, instrument configuration, and environmental factors—may impact test results. For this reason, it is strongly recommended that users establish or verify their own reference values under routine operating conditions, particularly when using this kit in a clinical diagnostic setting.
Diagnotix does not employ medically trained professionals and can only suggest possible ways to interpret results. Reference intervals may be culturally influenced, as vitamin levels can be affected by diet. Therefore, it is recommended to conduct independent reference studies when using this kit for clinical diagnostics.
Always consult a trained medical professional with expertise in the relevant field for the interpretation of results. The interpretation of test results also significantly depends on the individual characteristics of the patient. Diagnotix recommends considering these factors as well.

6. Summary of Analytical Performance Characteristics

Analytical performance characteristics have been defined by validation of the assay according to IVDR parameters, and using EP Evaluator to extract statistical data from the acquired raw data.

Repeatability (Simple Precision)

The repeatability, or simple precision, was analysed by measuring a patient sample, Diagnotix Vitamin A & E control I and Diagnotix Vitamin A & E control III twenty times from one sample within two hours from each other. From these results the Coefficient of Variation (CV) is calculated and compared to the precision verification goal which in turn is calculated using the biological variation.

Sample	**Simple precision Vitamin A (CV%)**	**Simple precision Vitamin E (CV%)**
Diagnotix Control I	1.9	2.1
Diagnotix Control III	1.9	2.2
Patient Sample	0.5	0.7

6.1 Reproducibility (Complex Precision)

The Reproducibility, or Complex Precision, was analysed by measuring a patient sample, Vitamin A & E SKML control low and Vitamin A & E SKML control High in duplicate twice a day over the course of ten days. From these results the Coefficient of Variation (CV) is calculated and compared to the precision verification goal which in turn is calculated using the biological variation.

Sample	**Complex precision Vitamin A (CV%)**	**Complex precision Vitamin E (CV%)**
Diagnotix Control I	2.4	3.4
Diagnotix Control III	2.7	3.3
Patient Sample	2.6	3.4

6.2 Linearity

The linearity was analysed by preparing a series of incrementally increasing vitamin A & E concentrations. These samples were prepared on two separate days and measured in duplicate. The mean results were calculated, the linearity was verified and the upper limit of detection was calculated.

Analyte	**Linearity (µmol/l)**
Vitamin A	➢ 9

Vitamin E	➢ 145 *

* For Vitamin E the linearity determined is 145 µmol/l. As this will result in an overload for sensitive analysers the linearity has been set to 75 µmol/l by Diagnotix.

6.3 Limit of Blank

The Limit of Blank (LOB) was analysed by measuring a zero sample (calibrator 1) twentyfold and a non-zero sample (calibrator 2) fivefold. The LOB was calculated from the responses with EP Evaluator.

Analyte	**Limit of Blank (µmol/l)**
Vitamin A	0.033
Vitamin E	0.1

6.4 Limit of Quantification

The Limit of Quantification (LOQ) was analysed by preparing a series of incrementally decreasing known vitamins A and E concentrations. The samples for vitamin A were prepared and measured seventeen times and those for vitamin E were prepared and measured twenty-six times. The sample preparation had a variance (different analyst, pipet, day, reagent temperature and/or calibration) each time to simulate different days in a laboratory. The limit of quantification was calculated with EP Evaluator.

Analyte	**Limit of quantification (µmol/l)**
Vitamin A	0.052
Vitamin E	<0.9

6.5 Correlation (Comparison)

For the correlation fifty-three patient samples were measured by Diagnotix with the LC-MS/MS and HPLC. The datasets were analyzed in EP Evaluator.

Analyte	**Passes**
Vitamin A	Yes
Vitamin E	Yes

6.6 Carry-over

To verify that there is no carryover two samples were prepared. One low (Vitamin A: 0.35 nmol/l and Vitamin E: 4.5 nmol/l) and one high (Vitamin A: 3.48 nmol/l and Vitamin E: 54.6 nmol/l). The samples were divided into eleven low samples and ten high samples. The samples were measured in a particular order after which the datasets were analysed.

Analyte	**Passes**
Vitamin A	Yes
Vitamin E	Yes

6.7 Accuracy

The accuracy of the method was determined by participating in the subscription schemes from the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML). This organization gathers results from all contributing laboratories and establishes a consensus or average. This in turn is compared to the results from Diagnotix.

Analyte	**Accuracy**
Vitamin A	Pass
Vitamin E	Pass

6.8 Matrix Effect

Matrix effects are addressed through use of stable isotope-labelled internal standards and matrix-matched calibrators and controls. No separate matrix effect study is therefore required.

6.9 Interferences

For Alpha-tocopherol, several structurally related vitamin E analogues may exhibit similar mass transitions, but the mass difference is at least 1 Da, which is large enough to distinguish between in α-tocopherol LC-MS/MS analyses:

Molecular Properties of Vitamin E Forms

Compound Name	**Molecular Weight (g/mol)**	**logP**
Alpha-Tocopherol	430.7	~9.2
Beta-Tocopherol	416.7	~8.9
Gamma-Tocopherol	416.7	~8.8
Delta-Tocopherol	402.7	~8.3
Beta-Tocotrienol	410.7	~8.8
Gamma-Tocotrienol	410.7	~8.7
Delta-Tocotrienol	396.7	~8.2

logP values indicate the lipophilicity of these compounds. Tocopherols and tocotrienols with logP values greater than 8 are highly non-polar and therefore elute under high-organic mobile phase conditions. This behavior causes all Vitamin E-related compounds to elute in a narrow retention time window, making chromatographic separation more challenging. However, because LC-MS/MS provides compound-specific detection via mass transitions, co-elution is not problematic. As such, full chromatographic separation of all isoforms is not strictly required for accurate quantification using this method.

Justification for Not Performing Interference Studies with Lipemic, Icteric or Haemolytic Samples

No interference studies with artificially lipemic, icteric or haemolytic samples are needed. This is based on the analytical design and performance characteristics of the method, which incorporates organic solvent-based protein precipitation and isotopically labelled internal standards to ensure robustness across variable serum matrices.

The sample preparation involves a substantial dilution and protein denaturation step using organic solvent, followed by centrifugation to remove precipitated material. This effectively minimises the presence of endogenous macromolecules and potential interferences. Furthermore, the use of deuterated internal standards compensates for matrix-related effects such as ion suppression or enhancement during electrospray ionisation in mass spectrometry. Selective detection through Multiple Reaction Monitoring (MRM) ensures high analytical specificity, reducing the likelihood of signal contributions from non-target compounds. The method has demonstrated reliable performance across serum pools of variable appearance, without bias or loss of precision, in internal validation studies.
Nevertheless, it cannot be excluded that aspecific interactions within the patient sample—potentially related to the patient's condition, specimen integrity, or contact with collection materials—may lead to erroneous results in rare cases. As such, all specimens must be subject to **visual inspection** prior to analysis. Samples exhibiting unusual appearance (e.g. excessive turbidity, discolouration, or signs of haemolysis) should be **critically assessed**, and if necessary, **a new specimen should be obtained**.

Final interpretation of test results, including the assessment of sample suitability, should always be performed by a **qualified clinical laboratory specialist in clinical chemistry**, who also bears full responsibility for determining whether results are analytically and clinically valid in the given context.
This approach is consistent with IVDR Annex I (GSPR 9.1), ISO 15189, and applicable good laboratory practice.

General Interferences

Over time, LC-MS/MS signal intensity may gradually decrease due to the accumulation of phospholipids on the column and/or within the system. These matrix effects can lead to variability and potential signal suppression.
To mitigate these risks, the following strategies are recommended:

- Use of phospholipid removal plates or tubes prior to analysis (Recommended: Phenomenex Phree plates or tubes. (Part numbers: 96-well plate 8E-S133-TGB; 1ml tubes 8B-S133-TAK))
- Thorough flushing of the UHPLC system with high-organic mobile phase between batches

Both Vitamin A and Vitamin E are sensitive to interference from possibly phospholipids. To improve method robustness, it is particularly important to avoid early elution of Vitamin A, which is more prone to co-elution with phospholipid background. An optimal retention time is

approximately **2.0 minutes**, which allows sufficient separation from possible interferences in the background. Depending on the specific configuration and performance of the user's system, this may require adjustment of the gradient and/or column to meet these conditions.
For further technical support or method optimization, consult your kit supplier or contact **Techsupport@diagnotix.com**.

7. General guidelines for using LC-MS/MS

7.1 Maintenance

LC-MS/MS devices are delicate systems that can easily being contaminated. Some parts of the device needs to be replaced or can damage over time. These factors can influence your obtained results. Therefore it is recommended to keep a tight LC-MS/MS cleaning schedule and consult a professional for general maintenance.

Injecting samples derived from biological matrices (e.g., whole blood, serum, plasma, urine, saliva) can lead to contamination of the U(HPLC) system, the analytical column, and the ion source over time. There is a significant risk that contamination will progressively spread further into the LC-MS/MS system. Therefore, general maintenance and preventive measures are essential to ensure long-term performance and accuracy.

Consult your LC-MS/MS supplier what are the recommended cleaning procedures.

7.2 Optimalisation of your LC-MS/MS device

Every system, even those of the same type from the same manufacturer, can behave differently. Therefore, copying U(H)PLC and MS settings from this manual or any other system to your own may result in suboptimal measurements.

It is advisable to use the settings provided in this manual as a starting point and optimize the UHPLC settings accordingly. For MS settings, we recommend tuning your system, as fragmentation can vary depending on your specific system and environment. Please refer to section 7.2.1 for general tuning guidelines.

7.2.1 General guidelines for tuning

Note: Please refer to guidelines and settings provided to your specific instrumentation. Ensure you are completely aware of the tuning procedures of your system. If our guidelines are conflicting guidelines of your device, please follow the guidelines of your device. The information giving in this manual has to been seen as purely informative.

1. Use Diagnotix 10 µg/ml solutions or a single pure molecule solution. Tuning mixes of different compounds only allow you the possibility of checking given conditions, but avoid you possibly the change of finding optimal conditions per single compound.
2. Use only LC-MS grade solvents to prevent contamination and background noise.
3. Check the instructions of your LC-MS device for the optimal tuning range (typically in the range of 50 to 500 ng/ml).
4. Prepare a dilution in a separate clean vial with a solvent mixture of 50% methanol / 50% water or what is likely the dissolving mixture wherein the compound will dissolve and precipitate from the analytical column.

5. Ensure the LC-MS/MS device is thoroughly cleaned prior to use and is functioning properly.
6. Start with a highly diluted sample to avoid contamination due to overloading. MS/MS systems can contaminate quickly when injecting too concentrated tuning solvents. You may increase the concentration in small steps if the signal is too low.
7. Rinse the source extensively with methanol or acetonitrile before and after each measurement.

7.2.2 Event window recommendations

Best practice is to make your event window as close as possible to avoid any contamination into the MS/MS. Therefore it is advisable reduce the window time as much as possible.

Set the concentration of the internal standard at 1.

Select the most abundant transition for the calculation, and the second most abundant transition as control.

8. Change Log

Version Change	Section changed	What has been changed	Date
1. 4→ 2.0	Complete revision	Complete rework of manual for IVDR compliance	12-09-2025

9. References

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